5-HT_3 Antagonists Derived from Aminopyridazine-type Muscarinic M1 Agonists

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A conformational analysis, performed on muscarinic M_1 agonists, identified four structural features characteristic of the muscarinic M_1 pharmacophore: (i) a protonable basic or quaternary nitrogen acting as a cationic head; (ii) an electronegative dipole usually part of a planar mesomeric ester, amide, or amidine function which can be replaced by an ether (muscarine) or a dioxolane (AF 30); (iii) an intercharge distance of 5 ± 0.5 Å between the cationic head and the electronegative atom of the dipole; (iv) an elevation of 0.5 ± 0.03 Å of the cationic head over the plane containing the electronegative dipole. During a reinvestigation of the conformational behavior of published structures of 5-HT_3 antagonists, similar features were observed for the 5-HT_3 pharmacophore. However many 5-HT_3 antagonists possess additional aromatic planes not present in the muscarinic M_1 agonists. These observations brought us to predict the chemical modifications that would change muscarinic M_1 agonists into 5-HT_3 antagonists. Four of the predicted aminopyridazines were actually synthesized and submitted to testing. The observed IC_{50} values for 5-HT_3 receptor binding ([^{3}H] BRL 43694) ranged from 10 to 425 nM, whereas the affinities for the muscarinic receptor preparations ([^{3}H] pirenzepine) layed over 10 000 nM. In electrophysiological studies the two most active compounds **10** and **13** produced antagonist-like effects on the 5-HT receptor channel complexes responsible for the generation of the rapidly desensitizing ionic currents, and agonist-like effects on those responsible for the slowly desensitizing components.

During the search for cholinergic agonists suitable for a symptomatic treatment of Alzheimer's disease, we prepared some years ago a series of 3-aminopyridazines. One of the compounds prepared, the 3-((2-(diethylamino)-2-methyl-1-propyl)amino)-5-propyl-6-phenylpyridazine sesquifumarate (SR 46559A, **1**) was selected for further development.[1,2] Presently it undergoes clinical trials as muscarinic M_1 agonist devoid of the cholinergic syndrome.[3,4]

Molecular modeling studies performed on 3-aminopyridazine-derived muscarinic M_1 agonists[2,5] showed some striking similarities between the muscarinic M_1 *agonist* and 5-HT_3 *antagonist* pharmacophore models. This analogy prompted us to use the 3-aminopyridazine core also for the design of 5-HT_3 receptor antagonists.

Pharmacophore of M_1 Receptor Agonists

Starting from a set of 12 published muscarinic M_1 agonists, the so-called "active analogue approach"[6] led us to a pharmacophore model for muscarinic M_1 agonists.[2,5] The characteristics of this model can be summarized as follows (Figure 1, left side): (i) A protonable basic or quaternary nitrogen acting as a cationic head, this cationic head is preferably surrounded by a lipophilic environment as found in amines such as piperidine, diethylamine, *N*-ethylpyrrolidine, quinuclidine, nortropane. (ii) An electronegative dipole usually part of a planar mesomeric ester, amide or amidine function. However, it can be replaced by an ether function (muscarine) or a dioxolane (AF30). (iii) An intercharge distance of 5 ± 0.5 Å between the cationic head and the electronegative end of the dipole. (iv) An elevation of 0.5 ± 0.03 Å of the cationic head over the plane containing the electronegative dipole.

Compound **1** and the related 3-aminopyridazines have selective affinities for hippocampal M_1 receptors.[1−3] In these compounds the protonated basic nitrogen can mimic the quaternary ammonium of acetylcholine. In assuming that the amidine function in the 3-aminopyridazine ring, unprotonated at pH 7.4,[7] can function as a bioisostere of the acetylcholine ester function in, it becomes plausible that **1** can be recognized by the muscarinic M_1 receptors as an acetylcholine mimic (Figure 2). Earlier studies from our laboratory have already made a very successful use of the amidine function in the 3-aminopyridazine ring as a bioisostere of the amidinic function found in sulpiride-derived dopamine antagonists.[8]

Antagonists of the 5-HT_3 Receptor: Conformational Study

In a previous paper, Hibert et al. characterized the geometric elements accounting for 5-HT_3 antagonism:[9]

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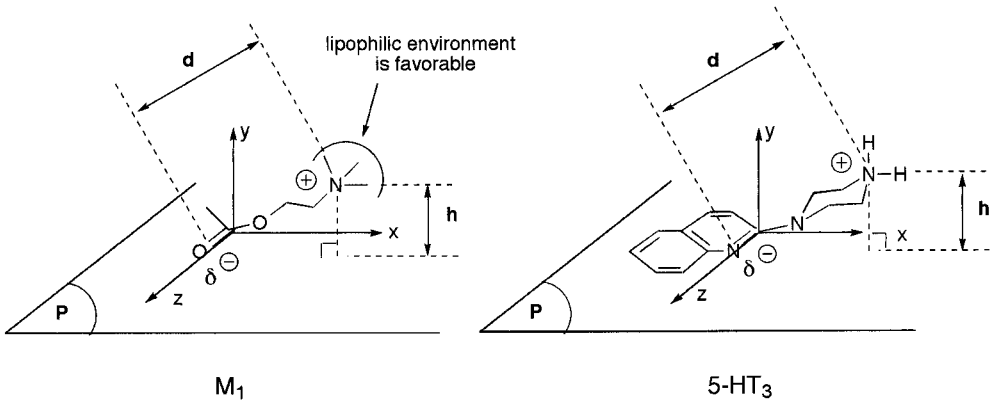


Figure 1. Pharmacophore model for muscarinic M_1 agonists applied to acetylcholine (left) and for serotoninergic 5-HT_3 antagonists applied to quipazine (right). Plane P contains the *x* and *z* axes and the O=C−O or the N=C−N functions.

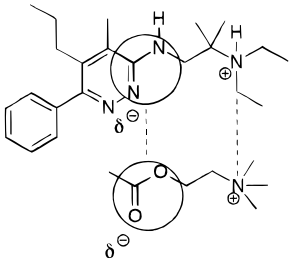


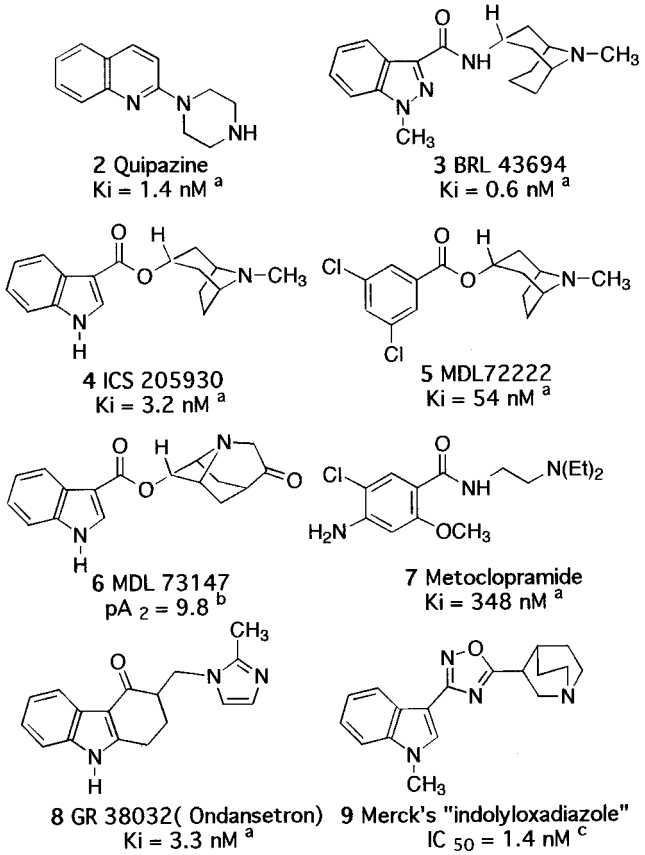
Figure 2. Bioisosterism between the side chain of compound **1** and acetylcholine.

(i) a basic amine, a carbonyl dipole (or an isosteric equivalent), and an aromatic plane coplanar to the carbonyl dipole; (ii) an intercharge distance of 5Å between the cationic head and the negative end of the carbonyl dipole; (iii) an elevation of 1.7 Å of the cationic head over the aromatic plane. However, a major drawback of this model is that, to locate the basic nitrogen at 1.7 Å above the quinoline plane, an axial attachment of the quinoline to the piperazine ring is implied. An equatorial conformation would certainly be more stable and actually is found in the crystal structure of other heterocyclic amidines related to quipazine.[10] For this latter reason we reinvestigated the conformational behavior of a set of known 5-HT_3 antagonists (Chart 1), using systematic search. In the present study, we focused only on low-energy conformers (5 kcal/mol energy window above the computed global minimum). The results of this conformational analysis are shown in Table 1.

In the conformational model of quipazine, there is a conformer presenting the geometrical characteristics found for muscarinic M_1 agonists (d = 4.8 Å and h = 0.48 Å) (Table 2). For each other compound, we looked for the existence of a conformer presenting similar geometrical constraints. These conformers were used and mapped rigidly on the "proposed bioactive conformation" found for quipazine (Figure 1, right side). Some typical superpositions are shown in Figure 3B.

It appears clearly that all of the compounds share a common recognition part represented by the tertiary amine, the dipole, and the aromatic system. However, when compared to the aromatic system found in quipazine, the aromatic rings of the other 5-HT_3 antagonists occupy different positions in space. Thus, when compared to quipazine (Figure 3A), the indolic benzo group of indolyl carboxylate **4** (green) occupies to the

Chart 1. Structure and Affinities of 5-HT_3 Antagonists[a]



[a] The biological data are taken from refs 9 and 11. (a) [^{3}H]GR65630 in cerebral cortex; (b) potency on rabbit heart serotonergic receptors; (c) [^{3}H]ICS205930 in cerebral cortex.

"northwestern" part of the molecule whereas the substituted phenyl group of metoclopramide **7** (magenta) is rather located in the "northeastern" part. These additional space occupations constitute possible extensions of the initial aromatic area found for quipazine.

3-Aminopyridazines as 5-HT_3 Antagonists

The comparison of the muscarinic M_1 agonist pharmacophore model with the one for 5-HT_3 antagonists guided us in the search for new potentially active 5-HT_3 antagonists. Thus, taking into account the previously defined geometrical elements, the 3-aminopyridazine **1** can be superimposed to quipazine in its serotononergic

Table 1. Conformational Analysis of Eight Representative 5-HT$_3$ Antagonists

	conformational analysis			selected active conformations		
compd	distance range[a] (Å)	elevation range[b] (Å)	no. of conformations	*d* (Å)[a]	*h* (Å)[b]	ΔE (kcal/mol)
2	4.7−5.2	−0.9 to 0.9	184	4.8	0.48	0.33
3	5.3−5.8	0.0−2.5	701	5.7	0.49	2.45
4	5.2−5.9	−2.2 to 3.2	580	5.3	0.49	4.17
5	5.1−5.8	−3.0 to 3.0	554	5.3	0.50	4.63
6	5.0−5.7	−2.9 to 2.9	625	5.3	0.50	4.00
7	2.9−5.3	−2.8 to 2.8	3033	5.2	0.47	1.79
8	4.0−4.8	−2.0 to 2.0	224	4.8	0.48	0.86
9[c]	2.9−5.3	−3.2 to 1.2	856	4.8	0.47	1.74

[a] Distance between the cationic head and the negative end of the dipole. [b] Elevation of the cationic head over the plane containing the dipole. [c] In ondansetron **9**, the cationic head is represented by a delocalized imidazolic system, therefore the centroid of N=C−N was used to map the cationic head.

"proposed bioactive conformation" as described above (Figure 3B).

These findings suggest that minor structural changes applied to aminopyridazines such as compound **1** should turn them into 5-HT$_3$ antagonists. Particularly, compounds **10**−**13** (Figure 4) can be expected to be valuable candidates for the switch from muscarinic M$_1$ agonists to 5-HT$_3$ antagonists.

They all have a relatively rigid and tertiary *N*-methylpiperazine side chain. According to the mapping of structurally diverse 5-HT$_3$ antagonists shown in Figure 3A, the different substitution patterns of the pyridazinic nucleus by an aromatic ring intend to explore the size of the aromatic area (Figure 3C).

Chemistry

The general procedure for the preparation of target compounds is shown in Scheme 1. Reaction of 3(2*H*)-pyridazinones **14**, **15** and 1(2*H*)-phthalazinones **16**, **17** with phosphorus oxychloride afforded the 3-chloropyridazines **18**, **19** and the 1-chlorophthalazines **20**, **21** respectively.

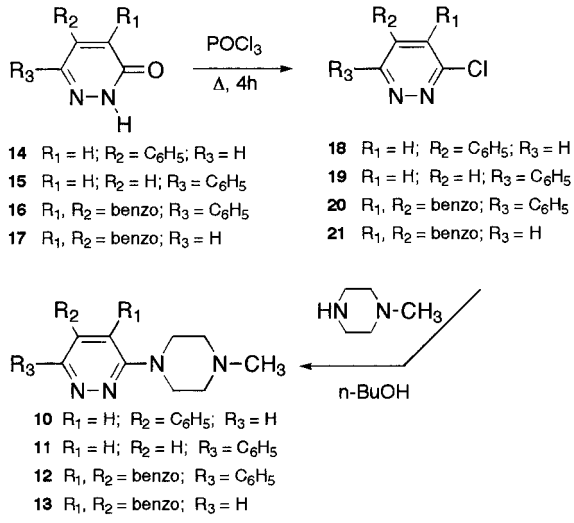
These compounds were then treated with *N*-methylpiperazine to yield the expected 3-(4′-methylpiperazin-1-yl)pyridazines or phthalazines **10**−**13** (Table 2). The starting 3(2*H*)-pyridazinones **14** and **15** or 1(2*H*)-phthalazinones **16** and **17** were prepared in condensing the appropriate γ-oxo carboxylic acid with hydrazine hydrate according to the literature procedures.[12−15]

Results and Discussion

The results from the radioligand binding assays at the 5-HT$_3$, M$_1$, and M$_2$ receptors are given in Table 2.

For the four compounds **10**−**13**, the observed IC$_{50}$ 5-HT$_3$ receptor binding values ([^{3}H]granisetron) range from 10 to 425 nM, whereas the affinities for the muscarinic receptor preparations([^{3}H]pirenzepine) lay over 10 000 nM. Thus, as predicted, compounds **10**−**13** show highly preferential affinities for 5-HT$_3$ receptor preparations. The factor which seems to be responsible for the 5-HT$_3$ preference, and which is common to the four compounds, is the relatively rigid tertiary *N*-methylpiperazinyl side chain. The lesser affinity of the 6-substituted pyridazines **11** and **12** suggests that a phenyl ring attached to position 6 of the pyridazine ring is rather detrimental. Concerning the agonist or antagonist profile toward the 5-HT$_3$ receptor preparations, the Hill number, close to 1 for all four compounds, suggests an antagonistic profile.

Scheme 1. Synthesis of the 3-(4′-Methylpiperazin-1-yl)pyridazines



14 R_1 = H; R_2 = C_6H_5; R_3 = H
15 R_1 = H; R_2 = H; R_3 = C_6H_5
16 R_1, R_2 = benzo; R_3 = C_6H_5
17 R_1, R_2 = benzo; R_3 = H

18 R_1 = H; R_2 = C_6H_5; R_3 = H
19 R_1 = H; R_2 = H; R_3 = C_6H_5
20 R_1, R_2 = benzo; R_3 = C_6H_5
21 R_1, R_2 = benzo; R_3 = H

10 R_1 = H; R_2 = C_6H_5; R_3 = H
11 R_1 = H; R_2 = H; R_3 = C_6H_5
12 R_1, R_2 = benzo; R_3 = C_6H_5
13 R_1, R_2 = benzo; R_3 = H

To confirm the antagonistic profile, and in order to establish the possible physiological properties of these molecules, a series of experiments on the dorsal root ganglion (DRG) neurons was then undertaken on the two most active compounds, **13** and **10**. DRG neurons express 5-HT$_3$ type of receptors on their membranes that are directly coupled with cationic channels.[16] This enables the ionic currents through the membrane, evoked by the rapid application of 5-HT, to be registered.

The population of DRG neurons is known to be heterogeneous, and not all neurons are 5-HT sensitive.[16] Besides, it is presently considered that 5-HT$_3$ receptors may be coupled with different cationic channels at different types of neurons.[17] An example is the biphasic (vs monophasic) rate of decay of currents resulting from the desensitization of receptor channel complexes during the sustained application of high concentrations of 5-HT.[17−19] In our experiments, 18 out of 26 tested neurons (69%) were 5-HT sensitive (5-HT: 50 μM). Among them, seven neurons revealed slowly desensitizing currents, nine neurons a biphasic desensitization profile in which the rapidly decaying component was followed by a slow component, and two neurons pos-

Table 2. Binding Affinities for the 5-HT$_3$, the M$_1$, and the M$_2$ Receptors of the 3-(4′-Methylpiperazin-1-yl)pyridazine and 1-(4′-Methylpiperazin-1-yl)phthalazine Hydrochlorides

compd no.	formula	5-HT$_3$[a] IC$_{50}$, nM (nH)[b]	M$_1$[c] K_i, nM	M$_2$[d] K_i, nM
10	$C_{15}H_{18}N_4 \cdot 2HCl \cdot 0.5H_2O$	36 ± 12 (1.35)	62000 ± 28000	80000 ± 9000
11	$C_{15}H_{18}N_4 \cdot 2HCl \cdot 1.5H_2O$	425 ± 87 (0.97)	15600 ± 970	71000 ± 7300
12	$C_{19}H_{20}N_4 \cdot 2HCl \cdot 0.5H_2O$	370 ± 73 (0.91)	13000 ± 240	116000 ± 10780
13	$C_{13}H_{16}N_4 \cdot HCl$	10 ± 3 (1.29)	10000 ± 3000	38000 ± 3200

[a] 5-HT$_3$ receptor ligand: [^{3}H]granisetron. [b] Hill number. [c] M$_1$ receptor ligand: [^{3}H]pirenzepine. [d] M$_2$ receptor ligand: [^{3}H]NMS.

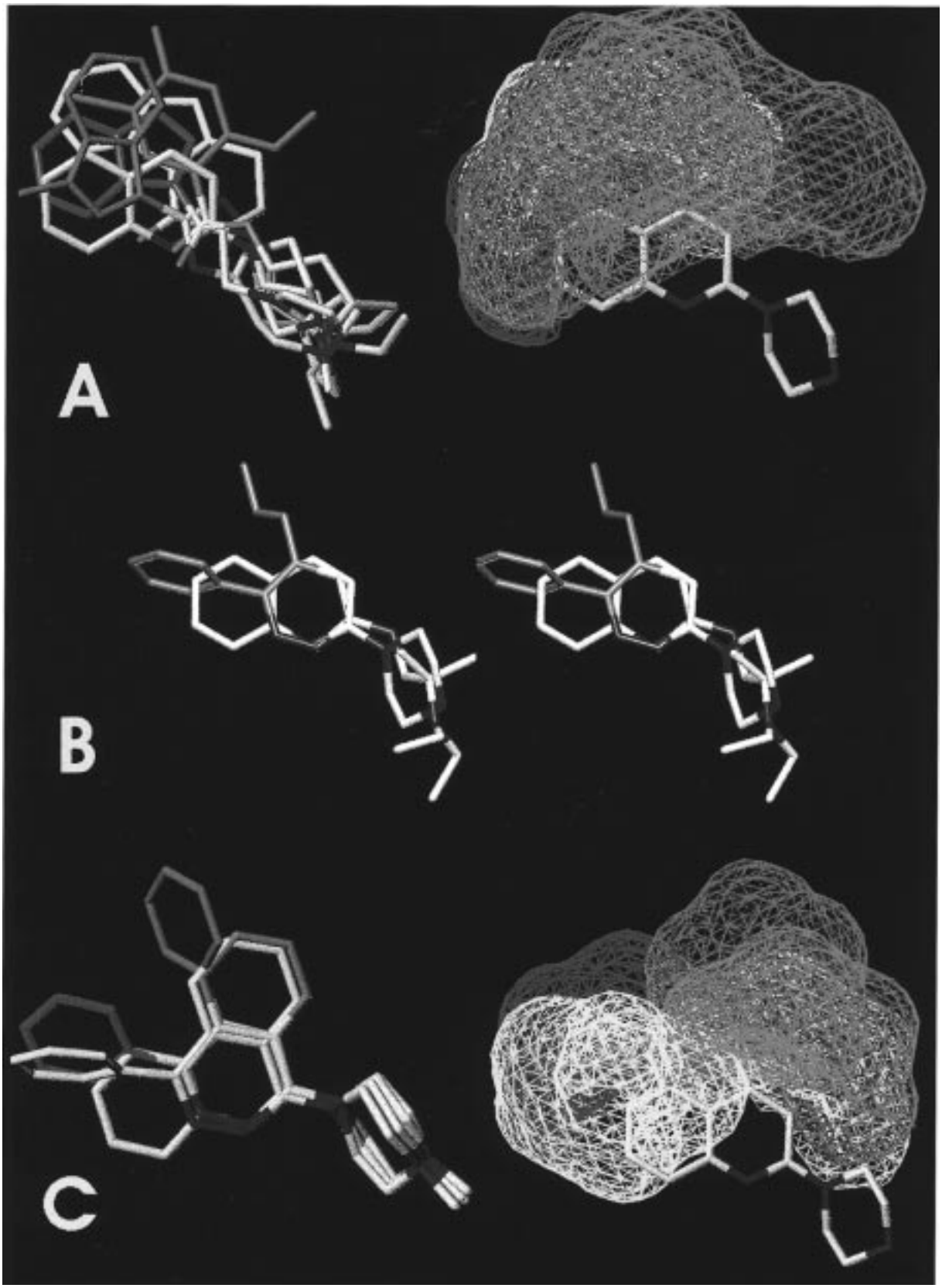


Figure 3. Molecular modeling. All molecules were superimposed (FIT program of the SYBYL software) using the *N*-methyl nitrogen atom of the piperazine ring and the two oxygen atoms of the ester function (or the corresponding atoms of the ester bioisoteres) as fitting points. The molecular volumes are displayed as Conolly surfaces (MOLCAD). (A) Overlay of compounds **4**, **7**, **8**, **9**, and quipazine (white). The aromatic extensions are coded as follows: **4** (green), **7** (magenta), **8** (yellow), **9** (red). (B) Overlay of quipazine (white), a 5-HT_3 antagonist, and the 3-aminopyridazine **1** (white and red), a muscarinic agonist (stereoview). (**C**) Overlay of the four 3-aminopyridazines **10**−**13** and quipazine (white). The aromatic extensions are coded as follows: **10** (red), **11** (green), **12** (yellow), and **13** (magenta).

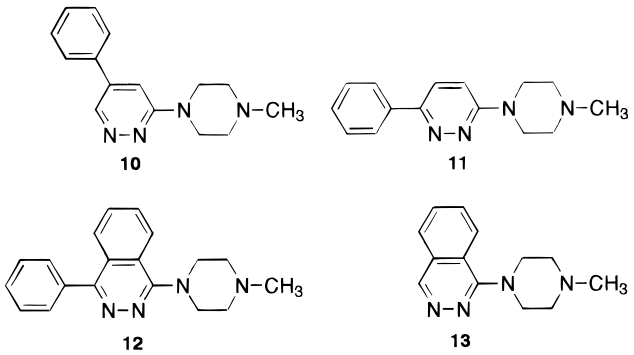


Figure 4. 3-Aminopyridazines predicted to be active as 5-HT$_3$ antagonists.

sessed only the rapidly desensitizing component of the current. At all 5-HT sensitive neurons, both the phthalazine **13** and the 5-phenyl analogue **10** produced clear pharmacological effects. In 10 μM concentration, both compounds mimicked the slowly desensitizing component of the current at the neurons at which this component could be evoked by 5-HT. In these cases coapplication of 5-HT (50 μM) during a sustained application of the phthalazine **13** and the 5-phenyl analogue **10** produced only negligible effects. At the neurons at which 5-HT evoked a biphasic character of receptor desensitization, both substances evoked only the slowly desensitizing component of current and blocked the rapidly desensitizing component of the current when 5-HT was coapplied. At the neurons at which an application of 5-HT (50 μM) evoked only the rapidly desensitized component of current, both of the ligands we tested (10 or 50 μM) did not produce appreciable currents and blocked them when simultaneously coapplied with 5-HT (50 mM). This kind of response was registered only at the small DRG neurons which are supposed to be C-type nociceptive neurons.

Summarizing the electrophysiological observations, it can be concluded that the phthalazine **13** and the 5-phenyl analogue **10** are effective modulators of the 5-HT$_3$ receptor functioning. They produce antagonist-like effects on the 5-HT receptor channel complexes responsible for the generation of the rapidly desensitizing ionic currents and agonist-like effects on those responsible for the slowly desensitizing components. A similar duality of pharmacological properties has recently been reported even for quipazine, which is classically referred to as a 5-HT$_3$ receptor blocker, and which is able to activate receptors in NG108-15 cells.[19] The physiological role of the rapidly and slowly desensitizing components of the neuronal responses produced by application of 5-HT is presently not clear. Of particular importance is the ability of phthalazine **13** and 5-phenyl analogue **10** to produce opposite effects on the two components of 5-HT-evoked currents. This property may be used for the discrimination between different types of 5-HT$_3$ receptor channel membrane complexes, as well as for a more detailed classification of DRG neurons that express 5-HT$_3$ receptor complexes on their membranes.

Taken together our results confirm a certain degree of relationship between the 5-HT$_3$ and the M1 pharmacophores. They demonstrate the easy possibility, in the 3-aminopyridazine series, to switch from muscarinic M1 agonists to serotoninergic 5-HT$_3$ antagonists of nanomolar potency. In electrophysiological studies, the two most active compounds, **13** and **10**, produced antagonist-like effects on the 5-HT receptor channel complexes responsible for the generation of the rapidly desensitizing ionic currents, and agonist-like effects on those responsible for the slowly desensitizing components. The present findings highlight, once again, the utility of the 3-aminopyridazines in the design of ligands of the biogenic amine receptors.

Experimental Section

Chemistry. Melting points were determined with a Mettler FP62 apparatus and are uncorrected. All ^{1}H NMR spectra were recorded on a Bruker AC 200 instrument (200 MHz), and chemical shifts are reported in parts per million (δ) relative to Me_4Si for $CDCl_3$ and Me_2SO-d_6 solutions (DMSO-d_6). Signals are quoted as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Flash chromatography was carried out on silica gel (70−230 mesh ASTM). Elemental analyses are indicated only by the symbols of the elements; analytical results were within ±0.4% of the theoretical values.

I. Pyridazinones and Phthalazinones. The starting pyridazinones are already known and were prepared by literature procedures.

5-Phenyl-3(2*H*)-pyridazinone (14): yield 78% (EtOH); mp 193 °C (lit.[12] mp 194 °C); ^{1}H NMR ($CDCl_3$) δ 8.13 (1H, s), 7.58−7.54 (5H, m), 7.11 (1H, s).

6-Phenyl-3(2*H*)-pyridazinone (15): yield 81% (EtOH); mp 201.5 °C (lit.[14] mp 202 °C).

4-Phenyl-1(2*H*)-phthalazinone (16): yield 90% (*i*-PrOH); mp 241.5 °C (lit.[13] mp 236−237 °C); ^{1}H NMR ($CDCl_3$) δ 8.58−8.52 (1H, m), 7.88−7.76 (4H, m), 7.63−7.52 (5H, m).

1(2*H*)-Phthalazinone (17): yield 68% (EtOH); mp 185 °C (lit.[15] mp 183−184 °C); ^{1}H NMR ($CDCl_3$) δ 8.47−8.42 (1H, m), 8.18 (1H, s), 7.91−7.72 (3H, m), 1.64 (1H, s).

II. 3-Chloropyridazines and 1-Chlorophthalazines. The appropriate substituted 3(2*H*)-pyridazinone or 1(2*H*)-phthalazinone was treated with an excess of phosphorus oxychloride (10 mL for 1 g of compound). The mixture was heated at 85 ± 3 °C for 4 h, then carefully poured onto ice (200 g for 1 g of compound), and finally rendered alkaline with 20% NaOH. The crude 3-chloropyridazine or 1-chlorophthalazine was collected by filtration, washed with water, and dried under vacuum. It was purified by crystallization in ethanol or 2-propanol or by chromatography on silica gel using a mixture of hexane−ethyl acetate (1:1).

3-Chloro-5-phenylpyridazine (18): 61%, yellow crystalline powder (EtOH); mp 181 °C; ^{1}H NMR ($CDCl_3$) δ 9.385 (1H, J = 2 Hz, d), 7.71−7.64 (3H, m), 7.59−7.55 (3H, m).

3-Chloro-6-phenylpyridazine (19): 82%, white crystals (EtOH); mp 159 °C (lit.[13] mp 158 −160 °C); ^{1}H NMR ($CDCl_3$) δ 8.08−8.03 (2H, m), 7.84 (1H, J = 10 Hz, d), 7.73−7.52 (4H, m).

1-Chloro-4-phenylphthalazine (20): 79%, white crystals (EtOH); mp 158 °C (lit.[14] mp 160−161 °C); ^{1}H NMR ($CDCl_3$) δ 8.41 (1H, J = 8 Hz, d), 8.12−7.71 (5H, m), 7.60−7.55 (3H, m).

1-Chlorophthalazine (21): 28%, yellowish crystals (*i*-PrOH); mp 117 °C (lit.[20] mp 119−120 °C); ^{1}H NMR ($CDCl_3$) δ 8.35−8.29 (1H, m), 8.06−7.99 (4H, m).

IV. Substituted 3-(4-Methylpiperazin-1-yl)pyridazine and 1-(4-Methylpiperazin-1-yl)phthalazine Salts. The *N*-methylpiperazinyl derivatives **10, 11**, **12**, and **13** were prepared from the corresponding 3-chloropyridazines **18**, **19** and 1-chlorophthalazines **20**, **21**.

General Procedure. To a solution of the appropriate 3-chloropyridazine (5×10^{-3} mol) in 1-butanol (30 mL) was added 8×10^{-3} mol of *N*-methylpiperazine and 5×10^{-3} mol of ammonium chloride. The reaction mixture was refluxed for 48 h. The solvent was removed under reduced pressure, and the residue was diluted in 100 mL of water. After alkalinization with solid K_2CO_3, the mixture was extracted with ethyl acetate (EtOAc) The combined organic layers were treated with a 10% solution of citric acid in water in order to recover the

obtained base. The separated aqueous phase was then rendered alkaline with K_2CO_3 and extracted with EtOAc. After drying over Na_2SO_4 and evaporation of the solvent, the obtained crude free base was purified by chromatography on silica gel using a mixture of ethyl acetate−triethylamine, 98:2 (v/v).

Formation of the Hydrochloride Salts. To the free base (0.01 mol) dissolved in 10 mL of *i*-PrOH was added 2 mL (0.024 mol) of 37% HCl. The hydrochloride salt usually crystallizes on standing, eventually after addition of anhydrous ether. The collected solids were recrystallized in absolute EtOH or *i*-PrOH.

3-(4-Methylpiperazin-1-yl)-5-phenylpyridazine Dihydrochloride (10). The free base was purified by chromatography on silica gel and elution with a 8:2:2 (v/v) mixture of ethyl acetate−methanol and triethylamine: yield 22% of yellow needles; ^{1}H NMR ($CDCl_3$) δ 8.875 (1H, s), 7.65−7.49 (5H, m), 7.02 (1H, s), 3.875 (4H, J = 6 Hz, t), 2.73 (4H, J = 6 Hz, t), 2.48 (3H, s). Dihydrochloride (EtOH): 70%; mp 285 °C.

3-(4-Methylpiperazin-1-yl)-6-phenylpyridazine Dihydrochloride (11). The free base (89%) was recrystallized in *i*-PrOH: ^{1}H NMR ($CDCl_3$) δ 8.02−7.39 (6H, m), 6.98 (1H, J = 10 Hz, d), 3.74 (4H, J = 6 Hz, t), 2.37 (3H, s). Dihydrochloride (EtOH): 40%; mp 250 °C.

1-(4-Methylpiperazin-1-yl)-4-phenylphthalazine Dihydrochloride (12). The free base (61%) was purified by chromatography on silica gel and elution with a 8:2:2 (v/v) mixture of ethyl acetate−methanol and triethylamine: ^{1}H NMR ($CDCl_3$) δ 8.07 (2H, J = 7 Hz, d), 7.87−7.72 (3H, m), 7.60−7.52 (3H, m), 3.65 (4H, J = 5 Hz, t), 2.75 (4H, J = 5 Hz, t), 2.44 (3H, s). Dihydrochloride (EtOH): 90%; mp 255 °C.

1-(4-Methylpiperazin-1-yl)phthalazine Hydrochloride (13). The free base (46%) was purified by chromatography on silica gel and elution with a 8:2:2 (v/v) mixture of ethyl acetate−methanol and triethylamine: ^{1}H NMR ($CDCl_3$) δ 9.145 (1H, J = 2 Hz, d), 8.06−8.00 (1H, m), 7.90−7.76 (3H, m), 3.54 (4H, J = 5 Hz, t), 2.685 (4H, J = 5 Hz, t), 2.44 (3H, s). Hydrochloride (*i*-PrOH): 80%; needles; mp 150 °C.

Computer Graphics Study. The study has been performed using the SYBYL 5.4 package[21] running on a Vax Station 2000/E&S PS390 configuration. All the molecules were built by assembling standard fragments, and the resulting geometries were optimized by molecular mechanics (Tripos Force Field). Systematic conformational analysis was performed by rotating each rotatable bond with increments ranging from 1 to 15° depending upon the flexibility of the molecules (SEARCH option of SYBYL). Detailed descriptions of the pharmacophore construction are given in the previous papers.[2,5,9]

Biology. 5-HT$_3$ Receptor Binding Test. The radioligand binding studies were performed according to a slightly modified version of the technique described by Nelson and Thomas.[22] Cerebral cortical tissue was obtained from male Sprague−Dawley rats (Charles River, France) and homogenized (Polytron, 30 s at speed 7) in 10 volumes of cold 50 mM HEPES buffer (pH = 7.5). The homogenate was centrifugated (10 min at 48000*g*, at 4 °C). The obtained pellet was washed three times by resuspending it in 10 volumes of HEPES buffer (Vortex) and centrifugating again.The final pellet was suspended in 20 mL of the previous HEPES buffer so as to obtain about 2.5 mg of protein/mL and stored on ice until required.

The binding experiments were performed using [^{3}H]granisetron ([^{3}H]BRL 43694),[23] a high-affinity ligand for the 5-HT$_3$ receptors. In a series of 16 × 100 mm polypropylene tubes aliquots of 0.4 mL of the membrane suspension were incubated during 45 min at 25 °C, in the presence of 0.3 nM of [^{3}H]BRL 43694 (NEN, specific activity 61 Ci/mmol) and increasing concentrations of the test compounds, the final volume being adjusted to 2 mL with the HEPES buffer. For the measurement of the nonspecific binding, the incubations were performed in the presence of 100 nM of zacopride. The incubated suspensions were then individually filtered on Whatman GF/B filters (diameter: 2.5 cm) which were previously impregnated by means of a 2 h soaking in a 0.3% polyethylenimine solution. The filters were then washed two times with room-temperature HEPES buffer (7.5 mL), and the radioactivity on the filters was counted in a liquid scintillation counter (Tri-Carb 2200 CA; Packard Co., Ltd.). The specific binding was determined by subtracting the nonspecific binding (in the presence of 100 nM zacopride) from the total binding persisting on the filters measured by liquid scintillation counting.

Muscarinic Receptor Binding Assays. The cortical M_1 receptor was identified with the binding of [^{3}H]pirenzepine in homogenates. Adult rat hippocampus was dissected on an ice-cold block and homogenized (1:200, w/v) with a polytron in 50 mM sodium−potassium phosphate buffer (pH = 7.4), EDTA (1 mM), and 0.1 mM PMSF (phenylmethanesulfonyl fluoride). Competition between [^{3}H]pirenzepine (0.7−1 nM) and the unlabeled test compounds was measured in an assay volume of 1 mL of this buffer with 0.5 mg of tissue; after 2 h 30 min at 25°C, the bound ligand was separated from free ligand with filtration over 0.3% polyethylenimine-treated Whatman GF/C filters. Atropine (10 mM) was used to determine the level of nonspecific binding.

The brainstem M_2 receptor was studied with [^{3}H]NMS binding. Adult rat cardiac cells were dissected and homogenized (1:200, ww/v) with a polytron in 50 mM sodium phosphate buffer, pH 7.4. The competition essay was conducted with 0.2 nM [^{3}H]NMS, 0.5 mg of tissue, and various concentrations of unlabeled ligand in an essay volume of 1 mL at 37 °C for 1 h. The bound [^{3}H]NMS was separated from free by filtration over Whatman GF/C filters. Atropine (10 mM) was used to determine the level of nonspecific binding.

Electrophysiological Investigation. The experiments were carried out on the freshly isolated, dissociated, and kept in culture (2−10 h) DRG neurons from rats (10−14 days old). The procedures of isolation of neurons[24] and electrophysiological recording[25] (patch-clamp, whole-cell configuration) were standard. Recordings of the ionic currents of neurons were performed in the extracellular low-calcium solution, containing (mM) the following: NaCl (140), KCl (3), $CaCl_2$ (0.1), $MgCl_2$ (3), HEPES (8), glucose (10). Neurons were perfused with the intracellular solution, containing (mM) the following: KCI (140), $CaCl_2$ (1), EGTA (10), HEPES (10), MgATP (1). The membrane potential of voltage-clamped neurons was held at −80 mV.

The modified method for rapid application of substances on the neuron[26] was used, being necessary because of the extremely rapid time rate of activation and subsequent desensitization of receptors (200 ms range). Ionic currents activated by 5-HT and tested substances reached maximal amplitude within 35−50 ms for the rapidly desensitizing and 200−300 ms for the slowly desensitizing currents. Blocking effects of phthalazine **17** and 5-phenyl analogue **14** on the rapidly decaying 5-HT-activated currents were completely reversible after washout of the drugs.

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